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(71) Applicant *(for all designated States except US)*: LIFECELL CORPORATION [US/US]; 3606 Research Forest Drive, The Woodlands, TX 77381 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: LIVESEY, Stephen, A. [AU/AU]; 104 Napoleon Street, Eltham, VIC 3095 (AU).

(74) Agent: CAGLE, Stephen, H.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

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(54) Title: RECONSTITUTED SKIN

(57) Abstract

The invention of this disclosure relates to a method for producing composite skin comprising a dermis inoculated directly with mammalian cells. In the method of this invention a dermis is obtained, preferably an acellular dermis of xenogeneic or human origin. The cells used to inoculate the dermis are transferred directly or preselected for properties consistent with progenitor or stem cell. The cells are of xenogeneic or human origin. In the practice of the preferred embodiments of this invention cells inoculated on the dermis are allowed to propagate *ex vivo* in culture to increase their number. In the preferred embodiment of this invention the *ex vivo* culturing results in the expansion of a population of cells having progenitor characteristics. The cells used to inoculate the dermis, in the preferred embodiments of this invention are genetically modified *ex vivo*. In the most preferred method of this invention the dermis to be inoculated is of human origin. In this embodiment the cells are either autologous or allogeneic in origin or a combination of both. The inoculated human dermis of this preferred embodiment can be applied to a human or utilized *ex vivo* as a laboratory assay.

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RECONSTITUTED SKIN

This invention relates to methods for the development of a reconstituted skin composite for transplantation. These methods will lead to the production a reconstituted skin consisting of an intact, biological, acellular, dermal matrix in combination with epidermal cells which can serve as a replacement for full-thickness skin defects.

Currently, the standard grafting procedure for full thickness skin injury involves the use of autologous split thickness skin grafts (STSG). While this has been shown to be a life saving procedure, there is still a need for improvement with regard to donor site trauma and the final cosmetic and functional outcome of the original wound. In full-thickness skin injuries, STSG must provide both dermal and epidermal components at the wound site. When autologous donor sites are limited, the STSG must be meshed and expanded to allow coverage of the entire wound area. This meshed configuration leaves areas of the wound uncovered by both dermis and epidermis. The epithelial cells of the grafted epidermis will eventually migrate into and cover the interstices of this mesh pattern and thereby promote wound closure. However, as dermis is not a regenerative tissue, problems with scarring and contracture arise later when the grafts contract due in part to a lack of sufficient dermis.

Another approach when STSG donor sites are limited is to culture epithelial cells from a biopsy of fresh healthy skin. These cells can be expanded in culture to allow coverage of a much larger area than the original biopsy site. Research to investigate the conditions necessary to grow keratinocytes *in vitro* began to emerge in the late 1960's and early 1970's. This research was aimed at defining the culture conditions necessary to propagate non-transformed keratinocytes for extended periods *in vitro*. Pioneering studies in this area were performed by Rheinwald and Green. One of the most important findings in these early investigations was the use of mouse

fibroblast 3T3 feeder layers and culture medium supplemented with epidermal growth factor (EGF). EGF was found to be a powerful mitogen for keratinocyte growth and allowed these cells to be propagated for several passages *in vitro*. Several other additives including cholera toxin, insulin and insulin-like growth factor, bovine pituitary extract, hydrocortisone and fetal bovine serum, to name a few, have since been found to be important additives for keratinocyte culture. This research led to the development of a serum-free, fully defined medium (MCDB-153) developed by Boyce and Ham. Together, these findings have allowed researchers to propagate keratinocytes *in vitro* for 10+ passages.

The culture of epithelial cells from a skin biopsy involves the separation of the epidermis from the dermis, followed by dissociation of the cells present in the epidermis. This can be accomplished using one or a combination of the following enzymes and chemicals to separate the two different layers of the skin: Dispase, Thermolysin, trypsin, or ethylenediaminetetraacetic acid (EDTA). The separated epidermis is then incubated further in trypsin plus EDTA to dissociate the epidermis into a cell suspension. Alternatively, it has been shown that microdissection of hair follicles followed by trypsin/EDTA incubation will also led to the growth of a population of keratinocytes. The dissociated cells are then placed into culture medium with a combination of growth factors, presence or absence of serum, and presence or absence of irradiated or mitomycin treated mouse fibroblast. If the cells are then allowed to remain in culture to or exceeding confluence, they will form an intact sheet of keratinocytes. This sheet can then be released from the culture vessel by treating with enzymes such as Dispase which disrupt the attachment of cells to the substrate but do not disturb cell-cell contacts.

These intact sheets of autologous keratinocytes (referred to as cultured epithelial autografts or CEA) can be produced from a small biopsy obtained from the patient. The production of these sheets however requires weeks of culture time. Although initial interest and use of CEA technology was high, as long term results

became available it was evident that the lack of dermal replacement imparts significant limitations on this approach including low overall take rates, scarring, and immature basement membrane formation leading to fragility of the epidermis.

An additional alternative for covering extensive burn wounds is micromeshing or microskin grafting. When autologous donor sites are limited, the available STSG can be meshed and widely expanded (generally at a ratio of 4:1 or greater) or minced by passing the tissue multiple times in different orientation through a standard mesher. While studies have shown that widely meshed autografts can eventually close a large full-thickness skin wound, these grafts a) take a long time to re-epithelialize interstices of the meshed graft, b) result in a "cobblestone" appearance at the graft site and c) often lead to debilitating scarring and contracture. As is the case for CEA grafting, the lack of dermal replacement in these procedures presents significant limitations on the final cosmetic and functional outcome. The use of an acellular dermal matrix, with an intact basement membrane complex, in combination with this technique will allow for a better cosmetic and functional outcome at the wound site.

Epithelial cells which migrate from the microskin pieces onto the basement membrane of the acellular dermis will also eventually migrate under the microskin pieces causing them to be sloughed from the surface. This will result in a smoother graft surface devoid of the cobblestone appearance. The presence of the acellular dermal matrix will also decrease scarring and contracture at the graft site.

In order to be fully effective, a graft for full thickness burn wounds should have the following characteristics: a) replace both lost dermis and epidermis, b) not require extensive *in vitro* cell culture to produce the graft, c) deliver a persistent dermis and epidermis, and d) require only one surgery and thereby reduce patient morbidity and mortality and reduce costs as a result of shorter hospital stays.

The invention of this patent includes the use of an intact acellular dermal matrix in combination with epithelial cells to reconstitute a composite skin meeting these requirements.

Dermal Matrices and In Vitro Reconstituted Skin: One technique for producing reconstituted skin involves using deepidermized dermis (DED), which was first investigated by Prunieras et al.. This dermal matrix is generally produced by prolonged incubation (> 4 weeks) of human skin in phosphate buffered saline or repeated freezing and thawing of the skin which kills all of the cells of the dermis and epidermis. Results with this technique have been variable. Human trials have demonstrated poor take rates in skin wounds using this substrate.

Krejci et al. have examined acellular versus cellular human dermal substrates in the presence or absence of an intact basement membrane complex *in vitro*. They found that papillary dermis lacking fibroblasts but maintaining an intact basement membrane, and reticular dermis which had been repopulated with dermal fibroblasts were both good substrates for keratinocyte growth. These results indicate the importance of basement membrane and/or dermal fibroblasts for the production of an *in vitro* skin.

As stated above, there have been numerous studies which report the necessity of fibroblasts to support a fully differentiated epidermis. To date, the majority of dermal substrates have been composed of animal collagen gels in which human fibroblasts are seeded prior to keratinocyte culture. Upon addition of fibroblasts, the collagen matrix contracts to approximately two thirds its original size. Of particular interest regarding these interactions is the report by Krejci et al., which demonstrated that an acellular dermal substrate with the basement membrane intact was as effective as fibroblast repopulated reticular dermis in supporting reconstitution of an epidermis. An argument can be made that the basement membrane acts as a sink for growth factors, calcium and other as yet unidentified substances which are secreted by fibroblasts and keratinocytes. In this way, an optimally preserved acellular dermis with the basement membrane intact can support keratinocyte growth for some period of time. After these stores are depleted, fibroblasts may become necessary to

reconstitute these growth factors and maintain the epidermis. Consistent with this hypothesis is a report by Higounenc et al., which found that epidermis reconstructed on DED, while exhibiting morphological and biochemical characteristics very similar to *in vivo* skin, did not fully normalize until after grafting onto an animal.

Attempts have been made to produce a biosynthetic composite skin as reported by Bell et al.. This composite was composed of fibroblasts seeded into a lattice of bovine collagen which was allowed to contract prior to overlying with keratinocytes. These studies reported the production of a multilayered epidermis and the formation of some basement membrane components within 2 weeks. This work was the foundation for the production of Graftskin™ (Organogenesis Inc., Canton, MA). Graftskin is a reconstituted composite skin currently in clinical trials for use in chronic ulcer treatment.

In 1988 Boyce, et al. introduced a synthetic, biological dermal substrate similar to that developed by Bell. This substrate was composed of human fibroblasts in a component collagen-glycosaminoglycan matrix. After seeding this substrate with keratinocytes they noted increased production of laminin and Type IV collagen *in vitro*, and a thicker epidermis with increased cell layers. This composite was then compared to cultured epithelial autograft sheets (CEA) for covering full thickness wounds on nude mice. The composite grafts showed increased epidermal rete ridge formation and better adherence and long term maintenance than the CEA. Reports of human clinical application of this matrix however are lacking.

Hansbrough et al. reported on what was described as an improved composite graft over that previously described. This skin substitute consisted of human fibroblasts cultured into a synthetic polyglactin mesh. The composite was further developed at Advanced Tissue Sciences, La Jolla CA., and is now called Dermagraft™. The reported advantage of this substrate was that it could be absorbed by hydrolytic rather than proteolytic degradation. However, results in nude mouse studies failed to show enhanced healing characteristics over those mentioned

previously. Further, despite clinical application there are no reports of successful reconstitution of both dermis and epidermis in full-thickness burn injuries.

Acellular Dermis: The inventors have previously been granted a patent regarding the processing and production of an intact, acellular dermal matrix of human or porcine origin (AlloDerm® and XenoDerm™ respectively).

The processing and preservation method was designed to generate a transplantable biological tissue graft that specifically meets the following criteria:

(a) provides an extracellular protein and collagen matrix which can be remodeled and repaired by the host,

(b) provides an intact basement membrane for secure reattachment of viable endothelial or epithelial cells,

(c) does not elicit a specific immune response by the host,

(d) does not calcify, and

(e) can be easily stored and transported at ambient temperatures.

The dermal matrix processed in this manner has been shown to possess all of the major components of the basement membrane complex including collagens Type IV and VII and laminin. Further, the matrix has been shown to be effective as a graft for severe burn wounds by replacing lost dermis, allowing immediate infiltration of host fibroblasts and endothelial cells and allowing the use of a thinner autologous split-thickness skin graft (STSG) [as a source for keratinocytes] resulting in less trauma to the donor site.

Epidermis: The epidermis is a continually renewing tissue composed primarily of keratinocytes. As such, there are at least three functionally distinct types of keratinocytes in the epidermis: 1. stem cells (progenitors), 2. transient-amplifying cells (exhibit rapid proliferative growth but for only a limited time), and 3. post-mitotic cells (mature differentiated). In this scheme the stem cell is ultimately

responsible for all keratinocyte replacement in the epidermis, and therefore is essential for long term maintenance of the organ. Therefore, the epidermal stem cell is necessary for the long term persistence of a grafted epithelium.

The current literature regarding keratinocyte research contains several references to epidermal stem cells (as detailed below). Unfortunately, unlike the hematopoietic system, specific markers for the putative epidermal stem cell have not yet been identified. The epidermal stem cell has however been associated with several distinct physical and functional characteristics which set it apart from the other keratinocytes of the epidermis. These properties include: long cell cycle time; enhanced expression of integrins or other markers including specific cytokeratins; small cell size relative to other keratinocytes; and rapid attachment to basement membrane components. These properties can be used to develop a protocol for the isolation and enrichment of epidermal stem cells for producing a composite skin in combination with an intact acellular dermal matrix. The isolation of the epidermal stem cell is analogous to the methodology used for protein purification where its physical properties are known but the sequence of the protein is unavailable. Hence, instead of separation and isolation by molecular weight, charge, solubility or selective adsorption (as used for protein purification) we can selectively enrich for epidermal stem cells by taking advantage of differences in cell cycle time, cell size, integrin or cytokeratin expression and attachment criteria. The epidermal stem cells can be selectively tagged by taking advantage of their slow cycling time, followed by selective isolation of the tagged cells by differences in size, marker expression and/or selective attachment to different substrates. By taking advantage of the physical and functional properties ascribed to the putative epidermal stem cell we can enrich for these cells and thereby enhance the formation of a neoepidermis in a composite graft.

Stem Cell Enrichment by Attachment: Epidermal keratinocytes which attach most rapidly to basement membrane components have been shown to possess the highest

colony forming efficiency. Specifically, research reported by Jones et al. has shown that keratinocytes which attach to collagen type IV coated dishes in as little as 5 minutes have a higher colony forming efficiency than those which take longer to attach. The epidermal progenitor cells may be isolated by performing panning techniques using culture vessels coated with type IV collagen, fibronectin, laminin, or a combination of these coatings. Alternatively, panning techniques can be performed using the acellular dermal matrix which has an intact basement membrane containing laminin and collagens type IV and VII, in the correct three dimensional configuration. Alternatively, partial degradation of the basement membrane complex present on the acellular dermal matrix may be necessary to mimic a wounded scenario and hence activate keratinocyte proliferation on the matrix. The basement membrane can be partially degraded by enzymatic treatment with Dispase II or Thermolysin. Close attention must be given to the collagen present in the dermis to ensure that the integrity of the dermis is not compromised during these enzymatic treatments. Keratinocytes can then be seeded onto an area of the treated dermal matrix.

Selective isolation of the epidermal stem cells by cell size: In order to take advantage of this property of epidermal stem cells, keratinocytes can be separated by size using either density gradient centrifugation, unit gravity sedimentation, or sorted by size using a cell sorter.

Density gradient centrifugation has been accomplished with keratinocytes using a continuous colloidal silica (Percoll) density gradient. Using this technique it is reported that 3 fractions of keratinocytes can be isolated. This corresponds well with the three proposed types of keratinocytes present in the epidermis (stem, transient-amplifying and terminally differentiated). Alternatively, unit gravity sedimentation can be performed. This procedure has been used in different laboratories to separate proliferative and terminally differentiating subpopulations of keratinocytes. Freshly isolated keratinocytes are placed in a modified sedimentation

chamber, from which aliquots of cells are removed and examined for label retention as defined in the localization studies. The cells can be evaluated for a progenitor phenotype by colony forming efficiency assays (CFE) and growth in soft agar as an index for stem cell isolation. Some potential problems with these techniques include: a) the disaggregation to a single cell suspension must be very efficient to avoid cell clumps which would sediment at different rates, and b) the size differential between stem cells and transient-amplifying cells may be as small as 1-2 micrometers making effective segregation very difficult. If the epidermis is not efficiently disaggregated to single cells, clumps of cells may be filtered through sterile cotton or nylon mesh. Although the different keratinocyte subpopulations may be very close in size, these techniques provide some enrichment over non-selected populations.

Stem Cell Enrichment by Anti-proliferation: Another cell selection technique involves selective killing of rapidly dividing cells (a negative selection process). 5-Fluorouracil, an antimetabolite, has been used in other systems to kill rapidly dividing or metabolically active cells. In this system, 5-FU can be used during *in vitro* culture conditions to selectively kill transient-amplifying (rapidly dividing) cells which have a short cell cycle time, while sparing the epidermal stem cells which have a longer cell cycle. This can be accomplished by pulse dosing of 5-FU during culture of rapidly expanding keratinocytes. These conditions may include culturing in the presence of 1 X 10^{-7} M retinoic acid for 24 hours followed by addition of 1 X 10^{-5} M isoproterenol for an additional 24 hours.

Hyperthermic treatment of the skin (or keratinocytes in culture) has been shown to decrease cell death due to UVB (290-320 nm) exposure. A hyperthermic approach has been shown to be effective on murine bone marrow cells. Wierenga et al. report that more primitive marrow stem cells are extremely heat resistant when compared to more differentiated cells. Acute (0.5-1 hour) heat exposure (40-44° C) can be used to eliminate the more rapidly dividing keratinocyte populations.

Alternatively, other environmental manipulations can also provide a selection pressure for epidermal stem cells. Specifically, hypothermia and hypoxia, as resistance to such changes is consistent with the critical importance of maintaining the stem cell *in vivo*.

Stem Cell Enrichment by Sorting Techniques: Perhaps the most elaborate methods currently used to isolate different populations of keratinocytes involves cell sorting techniques. Using these techniques it has been reported that keratinocytes which express the highest levels of $\alpha 2\beta 1$ integrin have the highest colony forming efficiency. Fluorescence activated cell sorting (FACS) has been used in a number of different systems to isolate cell populations which express unique markers. FACS is dependent on a fluorescein-conjugated marker or antibody to label specific cells which are to be isolated. As mentioned previously, no specific marker has yet been identified for epidermal stem cells. A recent scientific journal article has however described a population of keratinocytes which express the cytokeratin K19. These cells also have some of the phenotypic characteristic ascribed to epidermal stem cells. Antibodies to cytokeratin K19 are commercially available and hence may be used for FACS of this population of keratinocytes.

Due in part to the current lack of a mechanism for the purification of epidermal stem cells and in part to the nature of keratinocytes to differentiate when placed into culture, ex-vivo expansion of epithelial stem has not yet been described. This problem has however been overcome in the hematopoietic system. By investigating the effects which several different growth factors have on the growth of hematopoietic stem cells, researchers were able to define culture conditions which maintain these stem cells in a primitive, non-differentiated state. Some of these growth factors included; platelet derived growth factor (PDGF), granulocyte-macrophage colony stimulating factor (GM-CSF) and various interleukins.

Isolation of epidermal stem cells followed by expansion: Isolation of epidermal progenitor cells may be followed by limited expansion of these cells prior to application to the dermal matrix. The aim is to induce these cells to divide so as to increase the number of progenitor cells available for seeding onto the acellular dermal matrix.

Little is currently known about the definitive switch mechanism which induces keratinocytes to enter the committed state of terminal differentiation. There are however some instances in the hematopoietic system which may provide insight to this phenomenon. Specific growth factors have been found to be necessary for the maintenance of hematopoietic stem cell culture including, PDGF, GM-CSF and various interleukins. Combinations of these factors have been used to expand hematopoietic stem cells in culture.

The epidermis has become recognized as one of the most active secretory tissues of the body. Keratinocytes have been found to secrete interleukins -1, -3, -6, -7, -8 and -10, colony stimulating factors granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF) and GM-CSF, arachidonic acid metabolites, metabolites of vitamin D_3, parathyroid hormone-related protein, collagenases, tissue inhibitor of metalloproteinases and tissue plasminogen activator, transforming growth factor-alpha (TGF-α), TGF-β, tumor necrosis factor- alpha (TNF-α), PDGF and intracellular adhesion molecule-1 (ICAM-1). This is still only a partial list, and highlights the complexity of growth regulation in the epidermis.

Primary keratinocytes exhibit a finite life span *in vitro*. Culturing conditions optimized to retain the epidermal stem cells theoretically would allow indefinite culture and expansion of these cells. In a practical sense for clinical use, it is beneficial to seed keratinocytes onto the acellular dermal matrix as quickly as possible. In order to facilitate this, a stimulatory signal which induces the stem cell to divide once or twice will have a dramatic effect on the final expansion ratio.

Isolated epidermal stem cells can be cultured in medium containing one or more of the following growth factors which have been shown to stimulate keratinocyte growth: platelet derived growth factor (PDGF), granulocyte-macrophage colony stimulating factor (GM-CSF) (both found to be necessary for hematopoietic stem cell growth), tumor necrosis factor-alpha (TNF-α), transforming growth factor-alpha (TGF-α) (both potent stimulators of keratinocyte growth) and keratinocyte growth factor (KGF). KGF, one of the more recently defined growth factors in the epidermis, is a novel member of the fibroblast growth factor family and has been shown to have a stimulatory effect on keratinocyte growth. These culture conditions may also include growth of the cells on a 3T3 fibroblast feeder layer.

EXAMPLE 1

PRODUCTION OF A RECONSTITUTED COMPOSITE SKIN USING DISSOCIATED KERATINOCYTES IN COMBINATION WITH AN INTACT ACELLULAR DERMAL MATRIX

In the preferred embodiment of this invention, keratinocytes isolated from a biopsy of fresh human skin are applied directed to an intact acellular dermal matrix which is then transplanted to a skin defect.

The biopsy of fresh skin would be transported in cell culture medium containing 10% fetal bovine serum, penicillin and streptomycin. The tissue is kept at 4 degrees centigrade and processed within 24 hours. The tissue is handled with sterile instruments, dissected to remove extraneous fat and tissue, and cut into strips of no greater than 4 mm in width. The skin may be deepidermized with various enzymatic agents including trypsin, Dispase, Thermolysin or ethlenediaminetetraacetic acid (EDTA). The optimum method involves incubation in Dispase II (2.4 units/ml) at 37 degrees centigrade for 1.5 to 2 hours with periodic vortex mixing, followed by a 30 minute incubation in 0.25% trypsin plus 1 mM EDTA also at 37 degrees centigrade. The supernatant is then pipetted into a separate vial, spun down to pellet cells and resuspended in growth medium. This medium is composed of a 3:1 mixture of DMEM:Ham's F-12 supplemented with 10% fetal calf serum, 5 g/ml insulin, 0.5 g/ml hydrocortisone, 10 ng/ml epidermal growth factor, 10 ng/ml cholera toxin, and 0.15 mM Ca^{++}. The cells would then be seeded onto the acellular dermal matrix and transplanted to the patient.

Several combinations involving the isolation of epidermal cells, *in vitro* culture and seeding of the acellular dermal matrix can be accomplish including:

I The acellular dermal matrix is transplanted to the patient days prior to seeding of epidermal cells. This allows the dermal matrix time to become revascularized prior to the application of epidermal cells.

II The skin biopsy can be processed as described in the preferred embodiment followed by an *in vitro* culturing period during which the cell numbers are increased to allow seeding of a larger area of the acellular dermal matrix prior to transplantation.

III The acellular dermal matrix is transplanted to the patient days prior to seeding epidermal cells propagated as in II above.

IV The isolated epithelial cells can be directly seeded onto the acellular dermal matrix followed by culturing and expansion on the acellular dermal matrix prior to transplantation. This is accomplished by rehydrating the dermal matrix with three washes of Hank's balanced salt solution (HBSS), and placing the matrix in a culture flask or dish with the basement membrane facing up. Isolated human keratinocytes are then seeded onto the dermal matrix (at approximate 5×10^4 cells per cm^2 of the dermal matrix) and allowed to stand undisturbed in a cell culture incubator for 24 to 48 hours before changing the media. After this period the medium is changed with fresh medium containing 10^{-7} M all trans-retinoic acid. After an additional 16-24 hours the medium is changed again with the further addition of 10^{-5} M +/- isoproterenol. After an additional 24 hours exposure there will be a confluent layer of keratinocytes across the dermal matrix. The composite graft can be transplanted at this point.

V The composite graft can be continued in *ex vivo* culture and raised to the air-liquid interface by the use of a raised culture surface (such as a metal screen) which allows medium to reach the composite only from below. This exposure of the upper surface of the graft will induce some of the keratinocytes to begin a program of differentiation resulting in the formation of a stratified epidermis. During the air-liquid culture period the medium can be supplemented with other chemicals or agents which have also been shown to induce stratification in epithelial cultures. These

agents include, but are not limited to, calcium chloride and sodium butyrate. The resulting composite graft will now contain a fully stratified epidermis. The composite may be transplanted at this point.

VI Alternatively, the epithelial cells can be cultured to produce a CEA sheet prior to application to the dermal matrix. This process involves culturing of isolated keratinocytes to or exceeding confluence, at which time they will form an intact sheet of keratinocytes. This sheet can then be released from the culture vessel by treating with enzymes such as Dispase which disrupt the attachment of cells to the substrate but do not disturb cell-cell contacts. The sheet of CEA can be transferred to the acellular dermal matrix using a carrier such as Vaseline gauze followed by transplantation.

VII The acellular dermal matrix is transplanted to the patient days prior to the application of CEA sheets produced as in VI above.

EXAMPLE 2

MICROSKIN GRAFTING IN COMBINATION WITH AN ACELLULAR DERMAL MATRIX

In the preferred embodiment of this invention, a small piece of autologous, split-thickness, fresh human skin is passed through a skin mesher, fitted with a continuous cutting blade wheel, 2 times, at a 90° angle to each pass. Alternatively the skin can be cut into small pieces of approximately 1-1.5 mm^2 using a sharp scalpel. These microskin pieces are then spread evenly across an area of the basement membrane surface of the acellular dermal matrix which is approximately 10-50 times the original area of the starting piece of skin. The composite graft is then transplanted

to the wound surface and covered with a sheet graft of cryopreserved, human allograft skin.

Several combinations of microskin grafting in combination with the acellular dermal matrix can be accomplished including:

I The microskin pieces are transferred to the acellular dermal matrix which has been transplanted to the patient days previously.

II The composite of the preferred embodiment is allowed to propagate in *ex vivo* culture at the air-liquid interface as described in EXAMPLE 1 prior to transplantation.

III The composite of the preferred embodiment is composed of microskin pieces derived from an allogeneic skin biopsy.

IV The allogeneic microskin pieces are transferred to the acellular dermal matrix which has been transplanted to the patient days previously.

V The composite in III is allowed to propagate in *ex vivo* culture at the air-liquid interface as described in EXAMPLE 1 prior to transplantation.

VI The composite of the preferred embodiment and those described in I-V are covered with a synthetic polymer membrane which is then overlaid with the cryopreserved, human allograft skin at the time of transplantation.

EXAMPLE 3

SELECTION OF EPITHELIAL CELLS WHICH EXHIBIT CHARACTERISTICS OF EPITHELIAL PROGENITOR OR STEM CELLS

In the preferred embodiment of this invention, epidermal stem cells would be isolated by using the acellular dermal matrix as a panning substrate. This takes advantage of the previously described characteristic of the putative epidermal stem cell to attach rapidly to type IV collagen. The basement membrane of the acellular

dermal matrix is composed primarily of type IV collagen. The entire epithelial cell suspension is incubated on the dermal matrix for 30 minutes. The matrix is then washed with a light stream of culture medium to wash away unattached cells and then transplanted onto the patient.

There are several configurations of this grafting scenario involving stem cell isolation, propagation, seeding of the acellular dermal matrix and transplantation. These techniques may include any of the following:

I Panning on the acellular dermal matrix as described in the preferred embodiment followed by release of the attached cells from the dermal matrix using trypsin and seeding onto acellular dermal matrix which has been transplanted onto the patient days previously.

II Isolation of the epidermal progenitor cells by mechanisms which separate due to differences in cell size, followed by seeding of the selected cells onto the acellular dermal matrix and transplantation.

III Isolation of the epidermal progenitor cells by mechanisms which separate due to selective attachment to culture dishes coated with various dermal matrix components (e.g. fibronectin, type I collagen, vitronectin, or various glycosaminoglycans), followed by release of the cells from the culture dish using trypsin, seeding onto the dermal matrix, and transplantation.

IV Isolation of the epidermal progenitor cells by mechanisms which separate due to selective killing of rapidly dividing cells using antiproliferative agents, followed by seeding of the selected cells onto the acellular dermal matrix and transplantation.

V Isolation of the epidermal progenitor cells by mechanisms which separate due to selective sorting of cells expressing specific markers, followed by seeding of the acellular dermal matrix and transplantation.

VI Isolation of cells using any of the mechanisms described in II-VI followed by seeding of acellular dermal matrix which has been transplanted days previously.

VII Isolation of cells using any of the mechanisms listed in II-V followed by *ex vivo* propagation of the cells prior to seeding of the acellular dermal matrix and transplantation.

VIII Isolation and propagation of cells as described in VII followed by seeding onto acellular dermal matrix which has been transplanted days previously.

IX Use of the mechanism described in VII whereby the cells are isolated, seeded onto the acellular dermal matrix and propagated on the dermal matrix prior to transplantation.

CLAIMS:

1. A method for producing composite skin comprising a dermis inoculated directly with mammalian cells.

2. A method in claim one, whereby said dermis is acellular and intact.

3. A method in claim one, whereby cells are delivered to the dermis as a micromeshed autograft or allograft.

4. A method in claim one, whereby cells are disaggregated from epidermis and applied to the dermis and transplanted.

5. A method in claims one and three whereby the cells are delivered to the dermal matrix which has been transplanted onto the patient days previously.

6. A method in claims one and four, whereby cells are disaggregated and preselected for properties consistent with a progenitor or stem cell prior to application to the dermal matrix and then transplanted.

7. A method in claim six whereby the cells are applied to an acellular dermal matrix which has been transplanted to the patient days previously.

8. A method in claim six, whereby such properties include one or more selected from; adhesiveness of cells, cell size, label retention (long cell cycle) high clonogenic activity, expression of certain cell markers, resistance to anti-mitotic chemotherapeutic agents, and resistance to thermal variations.

9. A method in claims four to eight, whereby cells are propagated *in vitro* prior to application to the dermis and transplanted.

10. A method in claim nine, whereby the cells are applied to an acellular dermal matrix which has been transplanted to the patient days previously.

11. A method in claims four to eight, whereby cells are applied to the dermis and then cultured *in vitro* on the dermal matrix prior to transplantation.

12. A method in claims six and eight, whereby cells are propagated *in vitro* and then applied to the dermal matrix and cultured further prior to transplantation.

13. A method in claims nine to twelve, whereby such expansion is achieved using one or a combination of stem cell specific factors selected from a group including Granulocyte Macrophage - Colony Stimulating Factor, keratinocyte growth factors, serum factors, Transforming Growth Factor - α and β and Platelet Derived Growth Factor.

14. A method in claims one to thirteen, whereby cells have been genetically modified *ex vivo*.

15. A method in claim fourteen, whereby such genetic modification is selected from a group consisting of one or more of the following:

- to secrete a locally acting factor;
- to secrete a systemically acting factor, engineered to overcome a specific cell defect;
- engineered to bypass a specific immune response
- to exhibit transient expression

- to exhibit stable expression

16. A method in claim eleven, whereby said culture is further manipulated to produce a multilayered, stratified epidermis prior to transplantation.

17. A method in claims one to sixteen, whereby the dermal matrix is of xenogeneic origin.

18. A method in claims one to sixteen, whereby the dermal matrix is of human origin.

19. A method in claims one to sixteen, whereby the cells are of xenogeneic origin.

20. A method in claims one to sixteen, whereby the cells are of human origin.

21. A method in claim twenty, whereby the cells are either autologous or allogeneic in origin or a combination of both.

22. A method in claim twenty-one, whereby said inoculated dermal matrix is applied to a person.

23. A method in claim twenty-two, whereby said application of all inoculated acellular dermis takes place without prior propagation of cells in culture.

24. A method in claim twenty-two, whereby said inoculated dermal matrix is utilized *ex vivo* as a laboratory assay.

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PC1/US 96/13616

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 564 786 (LIFECELL CORP) 13 October 1993 see page 4, line 19 - line 53 see page 5, line 46 - line 55 see page 18, line 28 - line 40 see claims 28,33 ---	1-7, 9-12, 16-18, 20-23
X	WO,A,93 25660 (BRIGHAM & WOMENS HOSPITAL) 23 December 1993 see page 6, paragraph 3 - page 7, paragraph 1 see example 1 see claims 3-5,7-9,20,22,28-31 --- -/--	1,4,6-9, 14,15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 November 1996	06. 12. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Panzica, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 96/13616

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TRANSPLANTATION, vol. 60, no. 1, 15 July 1995, BOSTON US, pages 1-9, XP000196602 LIVESEY S.A. ET AL.: "Transplanted acellular allograft dermal matrix" see the whole document	1-3,5
X	THE JOURNAL OF INVESTIGATIVE DERMATHOLOGY, vol. 97, no. 5, November 1991, NEW YORK US, pages 843-848, XP000196611 KREJCI N.C. ET AL.: "In Vitro Reconstitution of Skin: Fibroblasts Facilitate Keratinocyte Growth and Differentiation on Acellular Reticular Dermis" see the whole document	1,2,4,9, 11,12, 18,19
X	JOURNAL OF DERMATHOLOGICAL SCIENCE, vol. 4, no. 3, November 1992, AMSTERDAM NL, pages 149-155, XP000196603 KREJCI N.C., MCGUIRE J.: "Treatment of burns with skin substitutes" see the whole document	1-5
X	WO,A,88 10296 (THE PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 29 December 1988 see page 9, line 28 - page 10, line 28 see claims 1,2,10,11	1,4,9, 17,24
A	US,A,5 292 655 (WILLE JR JOHN J) 8 March 1994 see abstract see column 12, line 40 - line 65 see column 23, line 23 - line 42 see column 2, line 66 - column 6, line 13	24

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 96/ 13616

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Please also see Further Information sheet under "reason".

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/ 13616

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark : Methods for treatment of human and animal body by surgery and therapy.

Incomplete Search :
Claims searched incompletely : 3-7, 9
Claims not searched : 22

Reason :
Claim 9 cannot depend on claim 5 nor claim 7.
Claim 11 cannot depend on claim 5 nor claims 7.

INTERNATIONAL SEARCH REPORT

..formation on patent family members

Interr nal Application No
PCT/US 96/13616

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EP-A-0564786	13-10-93	US-A-	5336616	09-08-94
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		AU-A-	3293493	19-08-93
		CA-A-	2089336	13-08-93
		JP-A-	6261933	20-09-94
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		AU-A-	4535693	04-01-94
		EP-A-	0644929	29-03-95
		JP-T-	8509356	08-10-96
WO-A-8810296	29-12-88	US-A-	4888291	19-12-89
		EP-A-	0321545	28-06-89
		JP-T-	2500564	01-03-90
US-A-5292655	08-03-94	NONE		